DEVELOPMENT OF A VALIDATED RP-HPLC METHOD FOR THE ESTIMATION OF CURCUMIN IN NANOEMULSION AND IN ITS PHASE SOLUBILITY STUDIES**

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The study developed a simple, sensitive and robust method for the estimation of curcumin in bulk drug form and in nanoemulsion formulation. Mobile phase consisting of acetonitrile and 20 mM potassium dihydrogen orthophosphate (pH 3.0) in ratio 55:45 was selected. A flow rate of 1 mL/min and an injection volume of 20 μL was injected to develop a chromatogram using a 516 C_{18} DB reversed-phase column. The detection wavelength was adjusted at 420 nm and the retention time of curcumin was found to be 9.78±0.5 min. The method was validated as per ICH guidelines and can be used for the quantification of curcumin even at a very low concentration.

Keywords: *RP-HPLC, curcumin, phase solubility, nanoemulsion.*

РАЗРАБОТКА МЕТОДА ВЫСОКОЭФФЕКТИВНОЙ ЖИДКОСТНОЙ ХРОМАТОГРАФИИ С ОБРАЩЕННОЙ ФАЗОЙ ДЛЯ ОЦЕНКИ КУРКУМИНА В НАНОЭМУЛЬСИИ И ЕГО ФАЗОВОЙ РАСТВОРИМОСТИ

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Разработан простой, чувствительный и надежный метод оценки куркумина в нерасфасованной лекарственной форме и в составе наноэмульсии. Подвижная фаза состоит из ацетонитрила и 20 мМ дигидроортофосфата калия (pH 3.0) в соотношении 55:45. Для получения хроматограммы вводили инъекцию со скоростью потока 1 мл/мин и объемом 20 мкл с использованием обращенно-фазовой колонки 516 C_{18} DB. Длина волны обнаружения 420 нм, время удерживания куркумина 9.78±0.5 мин. Метод соответствует рекомендациям ICH и может быть использован для количественного определения куркумина в очень низкой концентрации.

Ключевые слова: *высокоэффективная жидкостная хроматография с обращенной фазой, куркумин, фазовая растворимость, наноэмульсия.*

Introduction. Arthritis is a disorder of the joints that can lead to chronic disability. It is marked by inflammation, pain and destruction of the cartilage. The first line treatment for all forms of arthritis is with simple analgesics, steroidal and non-steroidal anti-inflammatory drugs (NSAIDs), followed by intra-articular injection of hyaluronic acid preparations and glucocorticoids as the disease progresses [1]. However, these treatments suffer from low efficacy, poor patient compliance and potentially harmful side effects [2]. Several anti-inflammatory drugs have been withdrawn by the FDA because of severe cardiovascular effects, and consequently, plant-based drugs are being promoted for the management of arthritis [3].

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Curcumin is a diferuloylmethane [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] found in turmeric (Curcuma longa, family Zingiberaceae) and has been used for centuries as a spice in the Indian subcontinent (Fig. 1) [4]. Curcuminoids are inhibitors of 5-lipooxygenase (LOX) and non-specifically cyclo-oxygenase (COX), resulting in its well-established anti-inflammatory action [5]. It has been reported to have a wide range of activities like anti-inflammatory, anti-oxidant, anti-parasitic, anti-mutagenic, anti-cancer and anti-arthritic (both in vitro and in vivo), as suggested by many researchers [6–12]. It has also been used in wound healing and as a dietary supplement for arthritis in traditional medicine. However, the wide use of this drug is limited by poor oral bioavailability due to extensive hepatic metabolism, low gastro-intestinal absorption and rapid elimination [13–16]. In previous studies, a lot of nanoformulation of curcumin have been developed and analyzed by various methods like UV-spectrophotometry [17], HPLC-tandem mass spectrometry [18], RP-HPLC [19], and HPTLC-DPPH method [20]. A RP-HPLC method was developed for the estimation of curcumin in nanoformulation and was validated as per ICH guidelines for linearity, accuracy, specificity, robustness, precision, limits of detection (LOD), and limit of quantitation (LOQ). The developed method was used to precisely identify and quantify the drug in oil, surfactant and co-surfactant during phase solubility studies for the development of nanoemulsion.

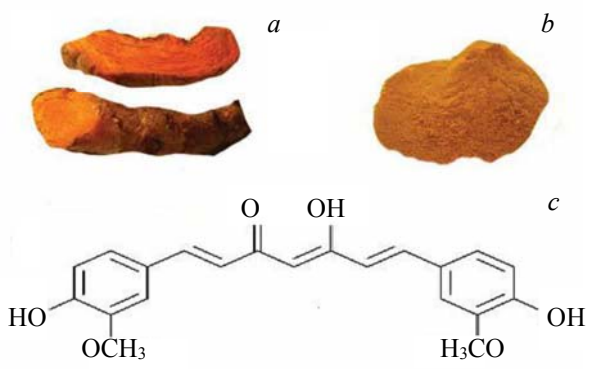

Fig. 1. Images of (a) turmeric rhizome, (b) curcumin powder and (c) chemical structure of curcumin.

Experimental. The system described is a Shimadzu model HPLC with quaternary LC-10A VP pumps, programmable variable wavelength UV/Vis detector, system controller SCL 10AVP (Shimadzu, Japan), column oven SPD-10AVP (Shimadzu, Japan) and a 20-μL loop Rheodyne injector. The software used was Class-VP 6.14.

To make a 20-mM buffer of potassium dihydrogen orthophosphate (KH_2PO_4), 2.721 g of potassium dihydrogen orthophosphate was dissolved in a small quantity of water and the volume was adjusted up to 1000 mL, whereas its pH was adjusted to 3.0 using orthophosphoric acid [21].

The reverse phase chromatographic column was used with specification 25 cm×4.6 mm internal diameter (ID) 516 C_{18} DB 5 μm (Supelco, Sigma-Aldrich, St Louis, MO, USA). The column and HPLC system were kept under optimum conditions. The mobile phase was 20 mM Potassium dihydrogen orthophosphate buffer (pH 3.0):acetonitrile in ratio 55:45 v/v, with a flow rate of 1.0 mL/min. The injection volume was 20 μL and elute was analyzed at a wavelength of 420 nm.

The experiment was conducted to determine the linearity between the concentration and the area under peak. A stock solution of CR was prepared by dissolving 50 mg of drug in 50 mL of acetonitrile to get a concentration of 1000 μg/mL. From the stock solution, 1 mL was withdrawn to a 10 mL volumetric flask and volume was made up to 10 mL using acetonitrile to obtain a 100 μg/mL of working solution. Different concentrations of CR from 200–800 ng/mL were prepared from a working solution 100 μg/mL and areas under peak were calculated. The mobile phase, after filtration through a 0.45-μm membrane filter, was delivered at 1.0 mL/min for column standardization, while the baseline was continuously monitored during the process. The wavelength of detection was selected at 420 nm. The prepared dilutions were injected serially and areas under the peaks were calculated for each dilution. All the readings were taken in triplicate.

The graph was plotted between concentration and area under the peak for linearity. Average asymmetric factor and retention time were calculated for each measurement. Asymmetric factor (AsF) is calculated as:

$$AsF = b/a,$$

where *b* is the distance from the point at the midpoint of the peak to the trailing edge, *a* is the distance from the leading edge of the peak to the midpoint. All the distances were measured at 10% of peak height.

The experiment used the standard addition method to determine accuracy. A single concentration of CR 400 ng/mL was spiked with 0, 50, 100, and 150% of the standard CR and the mixtures were analyzed by the proposed method. The experiment was performed in triplicate. The percentage recovery of samples and percentage relative standard deviation (% RSD) were calculated at each concentration level.

The ICH guidelines suggest that precision is considered at two levels: repeatability and intermediate precision. Repeatability was determined by carrying out intra-day and inter-day variations for the determination of CR at three different concentration levels of 200, 400, and 800 ng/mL in triplicates. For intermediate precision, the same sample was analyzed in a different laboratory on a different instrument.

To check the reproducibility of the method, system precision was obtained on a different instrument. A single concentration of 600 ng/mL was scanned six times and the area under the peak was calculated.

The LOD and LOQ were determined by the standard deviation ($\sigma_{y/x}$) method. A blank sample was injected in triplicate and its peak area was calculated. The determination of LOD and LOQ was done using the slope of the calibration curve and $\sigma_{y/x}$ of the blank sample by the following formulae:

$$\mathrm{LOD} = \frac{3.3\sigma\left(y/x\right)}{S},$$

$$\mathrm{LOQ} = \frac{10\sigma\left(y/x\right)}{S},$$

where $\sigma(y/x)$ is the standard deviation of the blank response, and *S* is the slope of the calibration curve.

Robustness was determined by changing the pH of the mobile phase to 2.8, changing the ratio of the mobile phase (50:50) and changing the flow rate (1.5 mL/min). These deliberate changes were made to find out the effect of variation in the chromatographic conditions for the determination of CR.

To select the most suitable oil, solubility studies were conducted. An excess quantity of the drug (CR) was added to 1 mL of different oils (viz. oleic acid, coconut oil, castor oil, sefsol, Labrafac PG, almond oil, isostearyl isostearate, olive oil, ethyl oleate, triacetin, arachis oil, sesame oil and Miglyol 810n taken separately in vials) until the loss of visual transparency. The vials were tightly stoppered and were continuously stirred for 24 h at 37±0.5°C on a shaker incubator (Shel lab, Sheldon manufacturing, Inc., USA). Samples were ultracentrifuged, and the supernatant was taken. The drug was extracted using liquid/liquid extraction with ethanol and the solubility was determined by the developed RP-HPLC analysis.

Similarly, for selection of the ideal surfactant Tween 80, Tween 60, Tween 20 and labrasol were tried. Likewise, PEG 400, plurol olique, propylene glycol and transcutol P were chosen as co-surfactants and the drug solubility was determined as described previously.

To find out the content of CR in nanoemulsion, 1 mL of nanoemulsion was concentrated over nitrogen gas to remove water and suitably diluted with the mobile phase to get 100 mL stock solution. This solution was sonicated for 10 min and analyzed for the drug content. The analysis was done in triplicate.

Results and discussion. A simple RP-HPLC method was developed and validated for the estimation of CR in the bulk drug form and nanoemulsion formulation. Shimadzu HPLC equipment, comprising of quaternary LC-10A VP pumps, SPD-10AVP column oven, a variable-wavelength programmable UV-Vis detector, and a SCL 10AVP system controller consisting of 25 cm×4.6 mm ID, 5-μm particle, 516 C_{18} DB reversed-phase column (Supelco, Sigma-Aldrich, St Louis, MO, USA), were used. The mobile phase was comprised of acetonitrile and 20 mM potassium dihydrogen orthophosphate (pH adjusted to 3.0) in the ratio 55:45 v/v, with a flow rate of 1 mL/min and injection volume of 20 μL. The detection wavelength was adjusted at 420 nm. The retention time of CR was found to be 9.78±0.5 as shown in Fig. 2. The concentration of the drug was calculated by comparing the area peaks of unknown and standard samples with known concentrations.

The linearity range for CR solutions was obtained by plotting area against concentration in the concentration range of 200–800 ng/mL as shown in Table 1 and Fig. 3. The linear regression data for the calibration curve showed a good linear relationship over the concentration ranges of 200–800 ng/mL, with respect to peak area with an r^2 value of 0.995: $y = 1051.2x+29995$, which is highly significant. No significant differences were observed in the slope of standard curves. The asymmetric factor was calculated at 10% of the peak height, which measures the tailing of the peak. The average retention time and asymmetry factor were found to be 9.709±0.13 min and 0.625±0.03, respectively. Values of linearity data, their standard deviation, %RSD, standard error and 95% confidence interval are shown in Table 1. All calculations were statistically done at a significance level of 5%.

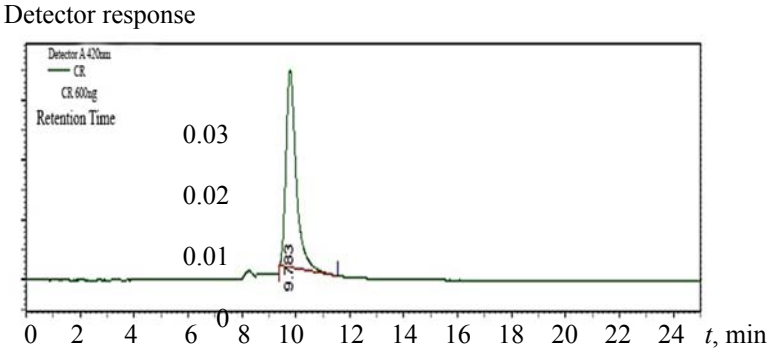

Fig. 2. HPLC chromatogram of curcumin (R_t 9.78 min).

TABLE 1. Linearity Data for Curcumin Using Peak Area (mean ± SD, n = 3)

Concentration, ng	Average area ±SD	% RSD	SE	95% Confidence limit	
				lower	higher
200	227586 ± 1081.61	0.48	624.49	224898.84	230273.164
400	475892.33 ± 3085.74	0.65	1781.61	468226.09	483558.58
600	647922 ± 3554.26	0.55	2052.11	639091.76	656752.24
800	871074.67 ± 6063.09	0.70	3500.63	856011.46	886137.87

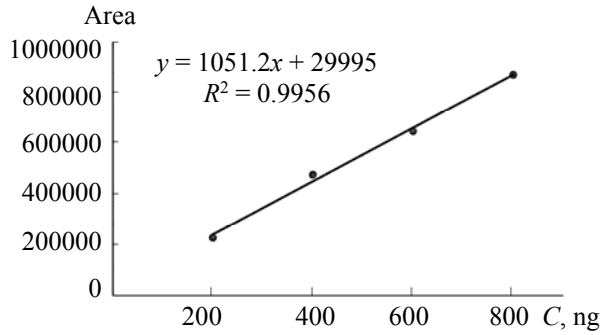

Fig. 3. Plot of curcumin for linearity.

The proposed method afforded recovery of 94.09–106.13% after spiking the additional standard drug solution to the previously analyzed test solution (Table 2). The % RSD was found to be less than 2, which signifies that the developed method can analyze the actual content very closely to the theoretical content.

Precision was considered at two levels of ICH guidelines, i.e. repeatability and intermediate precision. The repeatability of the analysis was determined as an intra-day and inter-day variation for the determination of CR at concentration levels of 200, 400, and 800 ng/mL in triplicates. Intermediate precision was determined by analyzing the samples in a different laboratory on different instruments. Results of repeatability and intermediate precision were expressed in terms of %RSD and are shown in Table 3. The low values of %RSD indicated the repeatability of the proposed method and there were no significant differences observed in the %RSD values of the intra-day and inter-day precisions.

The reproducibility of the method was checked by obtaining the precision of the method in another laboratory using different instruments and analyzed by another person. A single concentration of 600 ng/mL was analyzed six times and the results of reproducibility are follows: area = 6416922, 6437809, 6478056, 6497187, 6487142, 6476969, average area ± SD = 6465680.83±31266.76, and %RSD = 0.48.

LOD and LOQ of the method were determined by the standard deviation method and were found to be 18.15 and 55 ng/mL, respectively, which indicated that the proposed method can be used for the detection and quantification of CR even at very low concentrations.

There was no significant change in the retention time of CR by changing the ratio of the mobile phase and flow rate. A low value of the % RSD indicated the robustness of the method: concentration 400 ng, area 1 = 481290, area 2 = 479667, area 3 = 482765, average area ± SD = 481240.67±1549.58, and %RSD = 0.32.

TABLE 2. Accuracy as Recovery Study for Curcumin

Added, %	Concentration, ng	Average area ± SD	%RSD	Average content found ± SD	%Recovery
0	200	227801 ± 3132.64	1.38	188.17 ± 1.18	94.09
50	300	344546 ± 6530.84	1.01	299.23 ± 1.04	99.74
100	400	476253 ± 7389.27	0.85	424.52 ± 2.06	106.13
150	500	565865 ± 14046.68	1.29	509.77 ± 1.34	101.95

TABLE 3. Precision by Repeatability for CR Intra-Day Precision and Inter-Day Precision

Concentration, ng	Average area±SD (n = 3)	%RSD
Intra-day		
200	226561.5 ± 2233.75	0.99
400	468885 ± 4227.08	0.90
800	869597 ± 11996.77	1.38
Inter-day		
200	228456.5 ± 1448.86	0.63
400	469483 ± 4948.33	1.05
800	866261.5 ± 7114.20	0.82

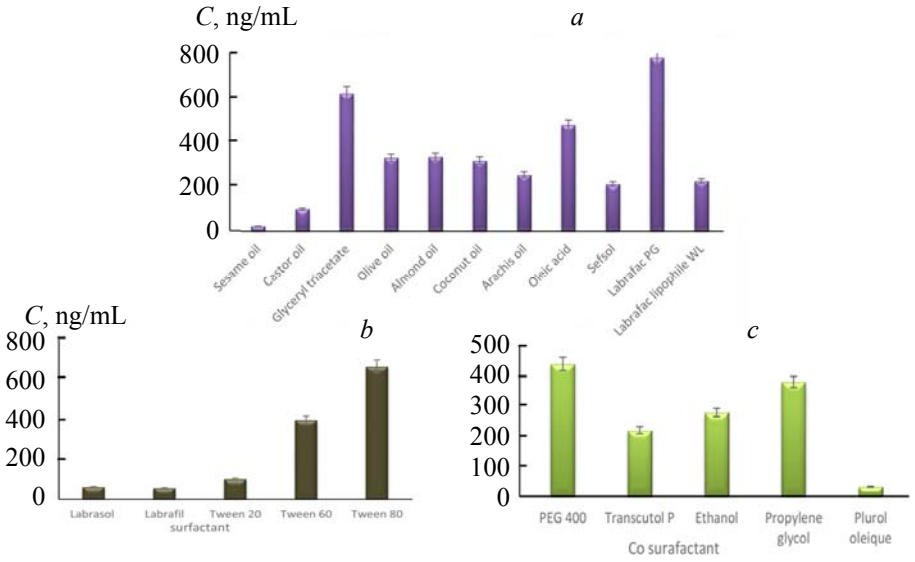

Fig. 4. Phase solubility study of curcumin in different oils, surfactants and co surfactants.

In the present study, different oils, natural as well as semi-synthetic, were chosen and the solubility of CR was determined as shown in Table 4 and Fig. 4. The selection of the oil phase was made in such a way that a maximum amount of CR should be incorporated and that it should be best suited for topical application. Therefore, the oil phase comprising Labrafac PG and glyceryl triacetate (triacetin) in equal ratio was selected. Glyceryl triacetate, being an excellent solvent blended well with the oil and enhanced the solubility of CR.

For the selection of surfactant and co-surfactant solubilization, efficiency was taken into account. The surfactant chosen must be able to lower the interfacial tension to a very small value, provide a flexible film that can readily deform around droplets and have the appropriate HLB value to provide the correct curvature at the interfacial region for the desired nanoemulsion type.

Transient negative interfacial tension and fluid interfacial film are rarely achieved by the use of a single surfactant, usually necessitating the addition of a co-surfactant [22]. Therefore, Tween 80, a non-ionic, non-irritant surfactant having HLB value of 15, was selected as the surfactant and PEG 400 was chosen as the co-surfactant as shown in Table 4 and Fig. 4.

TABLE 4. Determination of Solubility of CR (Phase Solubility Studies)

Oil	Average concentration ± SD, ng/mL
Sesame oil	20.86 ± 8.58
Castor oil	98.87 ± 13.19
Glyceryl triacetate	618.84 ± 10.62
Olive oil	329.48 ± 10.48
Almond oil	333.56 ± 6.25
Coconut oil	317.15 ± 2.23
Arachis oil	253.67 ± 10.50
Oleic acid	476.67 ± 17.90
Sefsol	211.33 ± 4.16
Labrafac PG	779.33 ± 8.96
Labrafac lipophile WL	224.67 ± 4.16
Surfactant	
Labrasol	60.32 ± 4.86
Labrafil	54.78 ± 2.01
Tween 20	99.65 ± 1.60
Tween 60	395.80 ± 16.18
Tween 80	662.21 ± 7.86
Co surfactant	
PEG 400	442.51 ± 22.86
Transcutol P	220.78 ± 2.08
Ethanol	280.38 ± 4.84
Propylene glycol	381.67 ± 12.37
Plurol oleique	32.30 ± 2.46

Nanoemulsion was formulated using Smix ratios of 4:1 (Tween 80: PEG 400) with an oil content of 12% v/v (Labrafac PG:Glyceryl triacetate = 1:1) and water. Curcumin at a concentration of 0.25% w/v was mixed thoroughly with the oil phase. Then, the required amount of *S* mix and water were added under constant magnetic stirring

The developed HPLC method was found to be suitable for the routine analysis of curcumin in pharmaceutical dosage forms. A single HPLC peak was observed at the same retention time from the nanoemulsion formulation. The content of curcumin was found to be 99.46% with a % RSD of 0.94. The low % RSD value indicated that degradation of curcumin had not occurred in the nanoemulsion formulation and that there was no interaction between curcumin and other excipients since pure curcumin was used and extracted from the nanoemulsion.

Conclusions. The developed HPLC method was found to be accurate, precise, reproducible and specific. It was as good as previously reported methods. The use of an accessible and economical mobile phase with a UV detector made it an excellent way for the quantification of curcumin in pharmaceutical dosage forms and also in bulk drug form. The solubility studies assisted in the production of nanoemulsion and the drug content in nanoemulsion was determined with a developed RP-HPLC method. The method is economical and easily available with no extraction procedures, low retention time, and no external standard was used or separate washing of the column was done. This makes it an excellent way for the quantification of curcumin in pharmaceutical dosage forms as well as in bulk drug form. The phase solubility studies helped in the development of nanoemulsion and the quantity of drug in nanoemulsion was estimated using the developed RP-HPLC method.

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